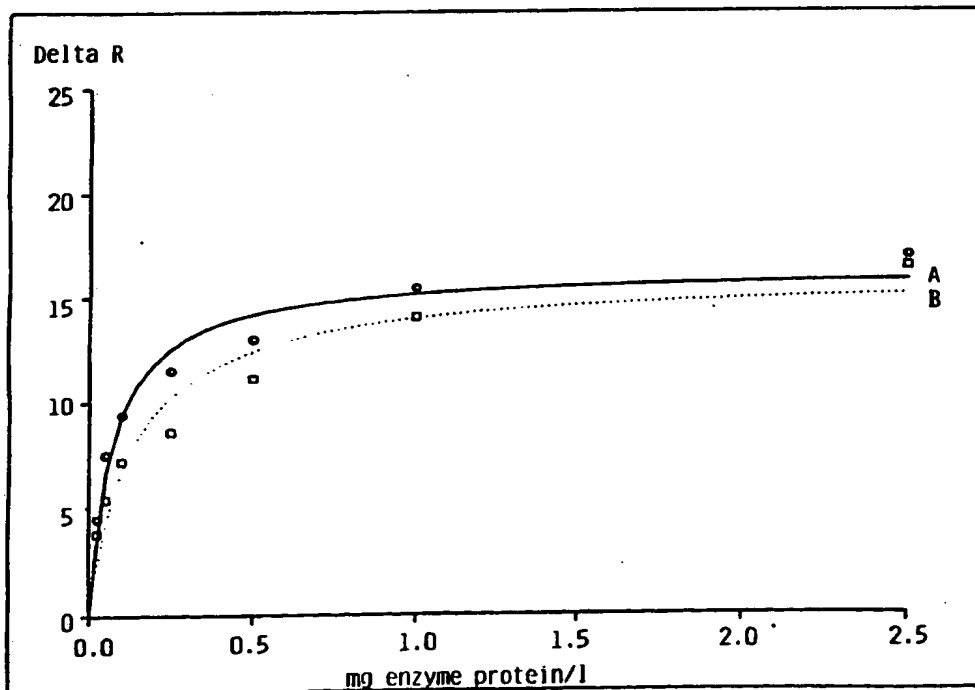




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(21) International Application Number: PCT/DK91/00342 (22) International Filing Date: 14 November 1991 (14.11.91) (30) Priority data: 2714/90 14 November 1990 (14.11.90) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : LINDEGAARD, Poul [DK/DK]; Øster Søgade 52, DK-2100 Copenhagen Ø. (DK). AASLYNG, Dorrit, Anita [DK/DK]; Fyrren 8, Svogerslev, DK-4000 Roskilde (DK). (74) Agent: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).		(81) Designated States: AT (European patent), BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), SU ⁺ , US. Published <i>With international search report.</i>

(54) Title: DETERGENT COMPOSITIONS**(57) Abstract**

This invention relates to detergent compositions. More specifically, the invention relates to detergent compositions containing alkyl sulphate and one or more subtilisins or subtilisin variants, which subtilisins or subtilisin variants either in position 195 hold a glutamic acid residue, or in position 222 hold an alanine residue, or both.

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DETERGENT COMPOSITIONS

TECHNICAL FIELD

This invention relates to detergent compositions. More specifically, the invention relates to detergent compositions containing alkyl sulphate and one or
5 more subtilisins or subtilisin variants, which subtilisins or subtilisin variants either in position 195 hold a glutamic acid residue, or in position 222 hold an alanine residue, or both.

BACKGROUND ART

Alkyl sulphate (AS) is an anionic surfactant used in various detergent
10 compositions, often in mixtures with other anionic surfactants, especially linear alkyl-benzene sulfonates (LAS). Anionic surfactants are known to provide excellent cleaning performance, and in numerous patent publications the use of AS as detergent ingredient is described, vide e.g. EP 219,314; EP 220,024; and EP 328,184.

15 In detergent compositions the use of AS is solely due to its superior detergency performance. It was certainly not to be anticipated that AS should have any positive influence on some detergent enzymes, while not on others. However, it has now surprisingly been found that AS exerts a positive influence on the performance of a specific group of detergent enzymes.

20

SUMMARY OF THE INVENTION

It has now been found that AS, when present, is able to increase the washability of certain detergent enzymes. These detergent enzymes belong to a

group of subtilisins holding a glutamic acid residue and/or an alanine residue at specific positions.

Accordingly, the present invention provides a detergent composition comprising alkyl sulphate and one or more subtilisins or subtilisin variants, which 5 subtilisins or subtilisin variants in position 195 hold a glutamic acid residue, and/or in position 222 hold an alanine residue.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which Figs. 1-3 show a comparison of the wash per-
10 mance of two detergent enzymes (A: Subtilisin 309/G195E/M222A; and B: Subtilisin 309) in three different model detergents (Model I-III, respectively, cf. the Example; Grass juice soiled cotton, 10 min. at 20°C, 3°dH, and pH 10).

DETAILED DISCLOSURE OF THE INVENTION

Experiments involving various detergent enzymes in various detergent
15 compositions have now demonstrated that certain subtilisins show increased wash performance in the presence of alkyl sulphate. More investigations on this subject indicated that a glutamic acid residue and/or an alanine residue at certain specific positions are responsible for this effect.

In respect to definitions of subtilisins, reference is made to e.g.
20 International Patent Publication Nos. WO 89/06279 and WO 91/00345. In these publications a survey of the amino acid sequence of various subtilisins is given. By alignment of the amino acid sequences of various subtilisins along with Subtilisin BPN' (Subtilisin NOVO) it is possible to allot a number to the amino acid residue position in any subtilisin, to the number of the analogous amino acid position in

Subtilisin BPN', and hence to indicate the position of an amino acid residue in a subtilisin enzyme unambiguously.

Thus, referring to this numbering system, in the context of this invention referred to as "BPN' numbering", the above mentioned investigations indicate that
5 a glutamic acid residue in position 195 and/or an alanine residue in position 222 (adjacent to the active serine at position 221) are responsible for the increased wash performance, when AS is present.

By a subtilisin variant, a mutated subtilisin, or a subtilisin derivative, is meant a subtilisin obtainable by alteration of a DNA nucleotide sequence of the
10 parent gene or its derivatives. The subtilisin variant or mutated subtilisin may be expressed and produced when the DNA nucleotide sequence encoding the subtilisin is inserted into a suitable vector in a suitable host organism.

The subtilisin variant, mutated subtilisin or subtilisin derivative may also be a subtilisin that by way of e.g. protein engineering or by chemical or biological
15 treatment is altered to produce a functionally equivalent derivative of said subtilisin, i.e. due to glycosylation or chemical stabilization.

In the context of this invention, when describing a mutation executed in a subtilisin, a specific nomenclature is adapted for ease of reference, the amino acid abbreviations being in accordance with the established one-letter code.
20 According to this nomenclature, the substitution of e.g. glycine with glutamic acid in position 195 is designated as "Gly 195 Glu" or "G195E". If the substitution is made by mutation in e.g. Subtilisin 309, the product is designated "Subtilisin 309/G195E". A product involving two mutations can thus be designated "Subtilisin 309/G195E/-M222A".

25 A detergent composition of this invention comprises alkyl sulphate (AS) and one or more subtilisins or subtilisin variants in which the subtilisins or subtilisin variants hold a glutamic acid residue in position 195 and/or an alanine residue in

position 222, and optionally the composition includes other detergent ingredients known in the art.

Within the scope of this invention are mutations or variants of the following subtilisins: Subtilisin BPN' (Wells et al. (1983); Proc. Natl. Acad. Sci. U.S.A. 84; 1219-1223; Wells et al. (1986); Phil. Trans. R. Soc. Lond. A. 317:415-423);
5 Subtilisin Amylosacchariticus (Kurihara et al. (1972); J. Biol. Chem. 247:5629-5631); Subtilisin 168 (Stahl and Ferrari (1984); J. Bacteriol. 159:811-819); Subtilisin Mesentericopeptidase (Svendsen et al. (1986); FEBS Lett 196:228-232); Subtilisin DY (Nedkov et al. (1985); Biol. Chem. Hoppe-Seyler 366:421-430); Subtilisin Carlsberg
10 (Jacobs et al. (1985); Nucl. Acids Res. 13:8913-8926); Subtilisin 147 and Subtilisin 309 (International Patent Publication No. 89/06279); Subtilisin Thermitase from Thermoactinomyces vulgaris (Meloun et al. (1985); FEBS Lett. 1983:195-200); Proteinase K (Betzel et al. (1988); Eur. J. Biochem. 178:155ff; and Gunkel et al. (1989); Eur. J. Biochem. 179:185ff); Aqualysin (Kwon et al. (1988); Eur. J. Biochem.
15 173:491ff); Bacillus PB92 Protease (European Patent Publication No. 283,075); Protease TW3 and Protease TW7 (Tritirachium album) (International Patent Application No. PCT/US88/01040).

In respect to mutations and methods of producing mutations in the subtilisin gene, reference is made to e.g. International Patent Publication Nos. WO
20 89/06279 and WO 91/00345; European Patent Application Nos. 130,756, 251,446, 328,229, and 415,296; and US Patent No. 4,760,025.

Preferred subtilisins or subtilisin variants are mutations of Subtilisin BPN' (Subtilisin NOVO); Subtilisin Carlsberg; Subtilisin 309; or Subtilisin 147, and/or variants hereof, i.e. Subtilisin BPN'/M222A; Subtilisin Carlsberg/M222A; Subtilisin
25 147/M222A; Subtilisin 309/G195E; Subtilisin 309/M222A; Subtilisin 309/G195E/M222A; and/or variants hereof. Most preferred is Subtilisin 309/G195E/M222A, and/or variants hereof.

By the term AS is meant a non-ethoxylated alkyl sulphate, being linear or branched, and containing from 1 to 20 C-atoms, more preferably 10 to 20, most preferably 12 to 16 C-atoms.

In a detergent composition of the invention, the alkyl sulphate constitutes at least 5%, preferably at least 15%, more preferred at least 25%, yet more preferred at least 35%, most preferred at least 50%, of the total amount of anionic surfactant present.

Besides AS the detergent composition of the invention may comprise additional surfactant(s), which may be of an anionic, non-ionic, cat-ionic, amphoteric or zwitter-ionic type, or a mixture of these. Typical examples of other anionic surfactants are linear alkyl benzene sulfonates (LAS); alpha olefin sulfonates (AOS); alcohol ethoxy sulfates (AES) and alkali metal salts of natural fatty acids. Examples of non-ionic surfactants are alkyl polyethylene glycol ethers; nonylphenol polyethylene glycol ethers; fatty acids esters of sucrose and glucose; and esters of polyethoxylated alkyl glucoside. In a specific embodiment of the invention, detergent compositions without any content of linear alkylbenzene sulfonates (LAS) are provided.

Other detergent ingredients known in the art include builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, stabilizers for the enzymes and bleaching agents, formulations aids, optical brighteners, foam boosters, chelating agents, fillers, fabric softeners, etc. The detergent composition of the invention may be formulated substantially as described in J. Falbe [Falbe, J.; Surfactants in Consumer Products. Theory, Technology and Application; Springer Verlag 1987, vide in particular the section entitled "Frame formulations for liquid/powder heavy-duty detergents"].

The detergent compositions of the invention can be formulated in any convenient form, such as powders, liquids, etc.

The detergent composition of the invention may advantageously include one or more other enzymes, e.g. lipases; amylases; cellulases; and/or peroxidases, conventionally included in detergent compositions.

The invention is further illustrated in the following example, which is not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE

This example relates to a comparison of the influence of AS on the wash performance of two detergent enzymes: Subtilisin 309 and Subtilisin 309/-G195E/M222A.

10 The wash performance tests were accomplished on grass juice soiled cotton in a model test, isothermally for 10 minutes at 20°C.

As detergents the following standard powder detergent compositions were used:

15 (mg)	Model I (mg)	Model II	Model III (mg)
AS (C ₁₂₋₁₈)	1230	615	-
LAS (NANSA 80 S)	-	615	1230
AES	107	107	107
20 Alfa-olefin-sulfonat	67.8	67.8	67.8
Soap	108.3	108.3	108.3
AE	64.3	64.3	64.3
Zeolit A	930.5	930.5	930.5
Sodium silicate	160.7	160.7	160.7
25 Na ₂ SO ₄	128.6	128.6	128.6
PEG 6000	64.3	64.3	64.3
Na ₂ CO ₃	546.4	546.4	546.4
	3407 mg	3407 mg	3407 mg

The detergent was dissolved in 4.5 litres of water of 3°dH (German Hardness), and the pH was measured to 10. The following enzyme dosages were used: 0.025; 0.05; 0.1; 0.25; 0.5; 1.0; and 2.5 mg protein/l.

The textile wash liquor ratio was approx. 6 g of textile per litre of wash liquor.

Subsequent to washing, the cloths were rinsed in running tap-water and air-dried. The remission (%R) was determined at 460 nm.

As a measure of the wash performance differential remission, ΔR was used being equal to the remission after wash with enzyme added, minus the remission after wash with no enzyme added.

The results of this comparative test are shown in Figs. 1-3 and in Table 1. In Table 1 the initial slope and the improvement factor (A/B) with respect to the initial slope are calculated.

Table 1

Detergent	AS/LAS-ratio	Initial slope		IF _s A/B
		A	B	
Model I	36% AS / - LAS	226.8	117.0	1.9
Model II	18% AS / 18% LAS	82.7	74.3	1.1
Model III	- AS / 36% LAS	33.3	57.5	0.6

A: Subtilisin 309/G195E/M222A

B: Subtilisin 309

IF_s: Improvement factor (in respect to initial slope)

The figures and Table 1 clearly illustrate the washability boosting effect of a detergent composition of the invention.

CLAIMS

1. A detergent composition comprising alkyl sulphate and one or more subtilisins or subtilisin variants, characterized in that the subtilisins or subtilisin variants in position 195 hold a glutamic acid residue, and/or in position 222 hold an alanine residue; the positions being in accordance with the BPN' numbering.
2. A detergent composition according to claim 1, characterized in that the subtilisins or subtilisin variants are Subtilisin BPN'/M222A; Subtilisin Carlsberg/-M222A; Subtilisin 147/M222A; Subtilisin 309/G195E; Subtilisin 309/M222A; or Subtilisin 309/G195E/M222A; or variants hereof.
- 10 3. A detergent composition according to either of claims 1-2, in which the alkyl sulphate constitutes at least 5% of the total amount of anionic surfactant present.
4. A detergent composition according to either of claims 1-2, in which the alkyl sulphate constitutes at least 15% of the total amount of anionic surfactant
15 present.
5. A detergent composition according to either of claims 1-2, in which the alkyl sulphate constitutes at least 25% of the total amount of anionic surfactant present.
6. A detergent composition according to any of claims 1-5, in which the
20 alkyl sulphate is a C₁₋₂₀-alkyl sulphate, more preferred a C₁₀₋₂₀-alkyl sulphate, most preferred a C₁₂₋₁₈-alkyl sulphate.

7. A detergent composition according to any of claims 1-6, in which no linear alkylbenzene sulfonates (LAS) are present.

8. A detergent composition according to any of claims 1-7, being a liquid detergent composition.

5 9. A detergent composition according to any of claims 1-7, being a powder detergent composition.

1/3

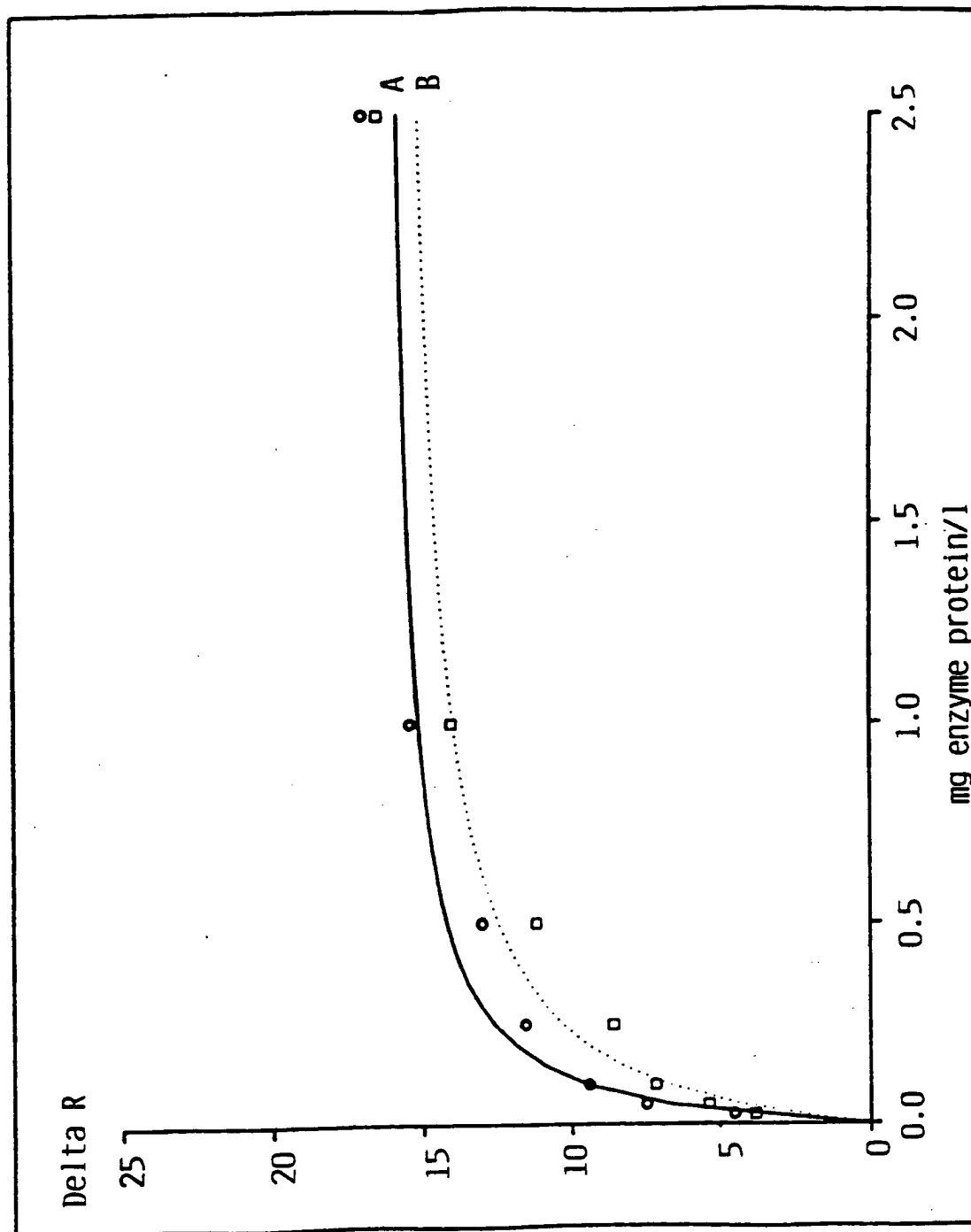


Fig. 1

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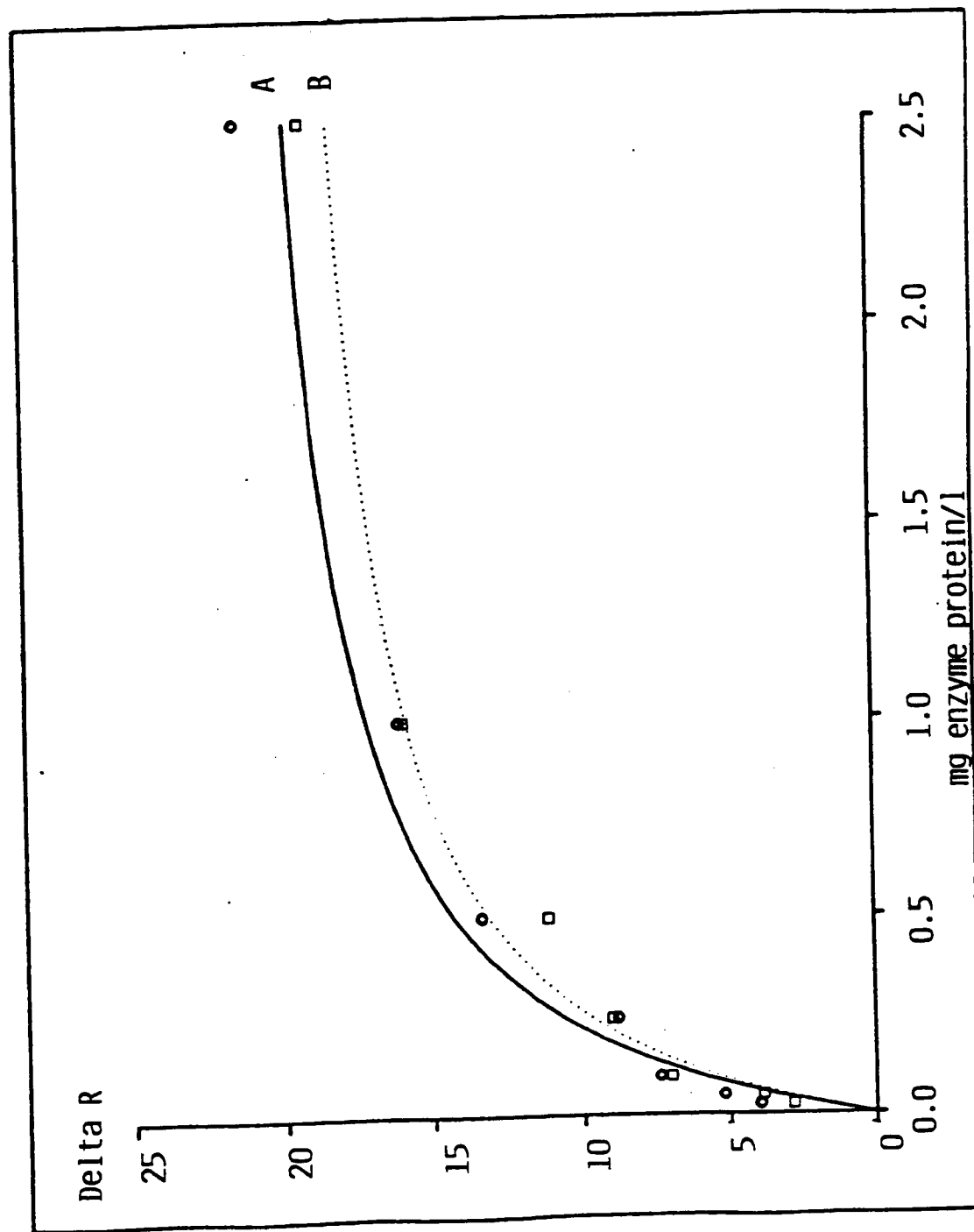


Fig. 2

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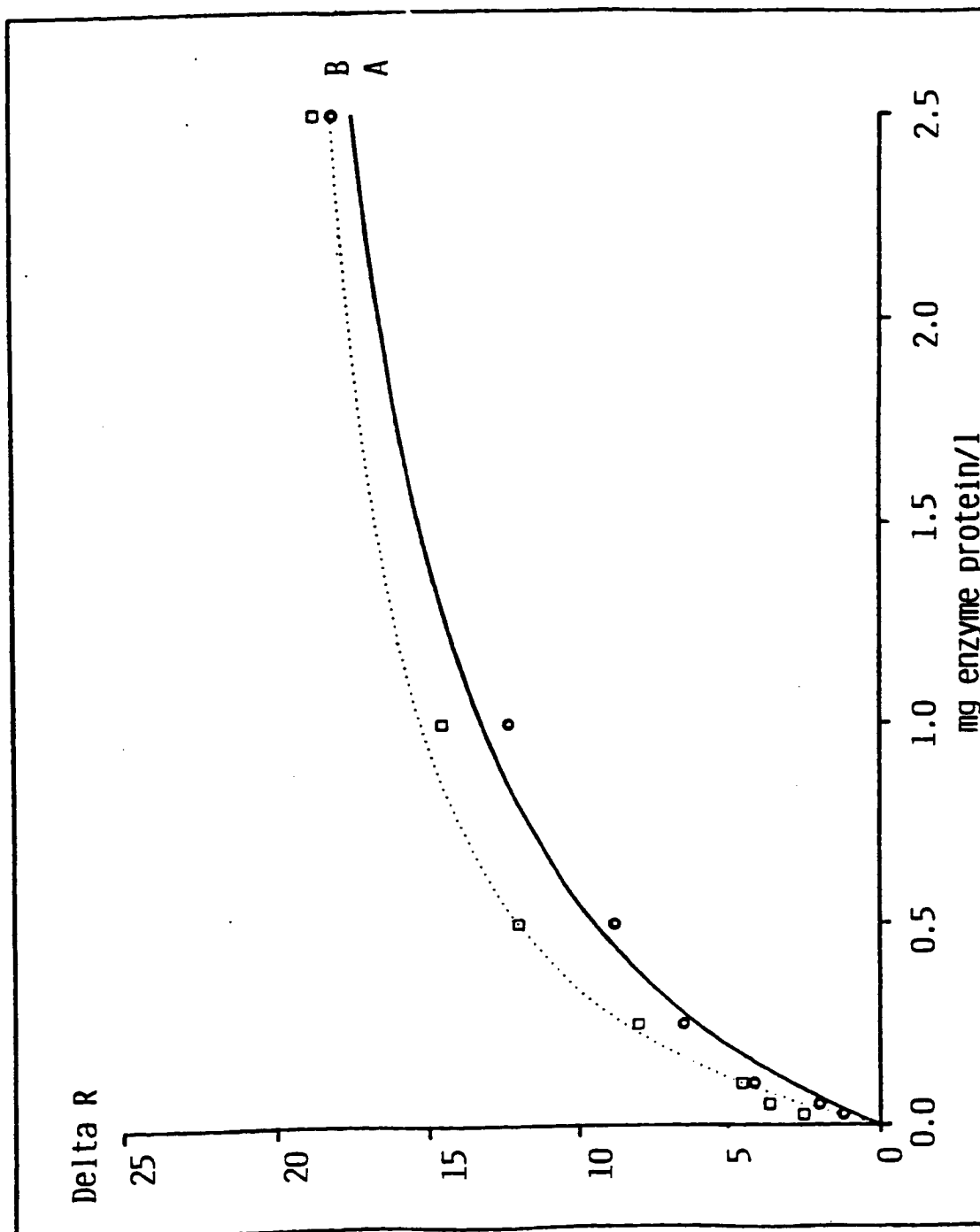


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No **PCT/DK 91/00342**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 11 D 3/386						
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 20%; border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification System</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification Symbols</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">IPC5</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">C 11 D; C 12 N</div> </td> </tr> </table>			<div style="border: 1px solid black; padding: 2px;">Classification System</div>	<div style="border: 1px solid black; padding: 2px;">Classification Symbols</div>	<div style="border: 1px solid black; padding: 2px;">IPC5</div>	<div style="border: 1px solid black; padding: 2px;">C 11 D; C 12 N</div>
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<div style="text-align: center; font-size: small;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div>						
SE,DK,FI,NO classes as above						
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹						
<div style="border: 1px solid black; padding: 2px;">Category *</div>	<div style="border: 1px solid black; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</div>	<div style="border: 1px solid black; padding: 2px;">Relevant to Claim No.¹³</div>				
X	WO, A1, 8906279 (NOVO INDUSTRI A/S) 13 July 1989, Abstract; page 3, lines 1-11; claims 1, 23, 33 and 36 <div style="text-align: center;">--</div>	1-9				
X	EP, A1, 0130756 (GENENTECH, INC.) 9 January 1985, fig. 7 and 10; page 20, lines 7-23 <div style="text-align: center;">--</div>	1-9				
X	EP, A1, 0328229 (GIST-BROCADES N.V.) 16 August 1989, Abstract; claims 1-3, 5-8, 33-35 and figure 4 <div style="text-align: center;">--</div>	1,8-9				
P,X	WO, A1, 9100345 (NOVO NORDISK A/S) 10 January 1991, Abstract; page 11, lines 16-36; page 26, line 16 - page 29; page 31, line 24 - page 34 <div style="text-align: center;">--</div>	1-9				
<div style="font-size: x-small;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> </div>						
IV. CERTIFICATION						
<div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div>		<div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div>				
<div style="border: 1px solid black; padding: 2px;">18th February 1992</div>		<div style="border: 1px solid black; padding: 2px; text-align: center; font-weight: bold;">20 FEBRUARY 1992</div>				
<div style="border: 1px solid black; padding: 2px;">International Searching Authority</div>		<div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div>				
<div style="border: 1px solid black; padding: 2px; text-align: center;"> SWEDISH PATENT OFFICE </div>		<div style="border: 1px solid black; padding: 2px;"> <div style="text-align: center;">Dagmar Järvmä</div> </div>				

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	EP, A1, 0405901 (UNILEVER PLC ET AL) 2 January 1991, page 17, line 13 - page 18, lines 1-58; page 19, line 45 - page 20, lines 1-39; claims 1-46 --	1-9
P,X	EP, A1, 0398539 (AMGEN INC.) 22 November 1990, examples 8-10; page 7, lines 12-51; claims 1, 16, 21-22, 25 --	1-9
A	US, A, 3790482 (THE PROCTER & GAMBLE COMPANY) 5 February 1974, see the whole document -- -----	1-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00342

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 30/12/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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